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The Anatomy and Morphology of the Adult Bacterial Light Organ of *Euprymna scolopes* Berry (Cephalopoda:Sepiolidae)

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Abstract. The sepiolid squid, *Euprymna scolopes*, has a bilobed luminous organ in the center of the mantle cavity, associated with the ink sac. Luminous bacterial symbionts (*Vibrio fischeri*) are housed in narrow channels of host epithelial tissue. The channels of each lobe of the light organ empty into a ciliated duct, which is contiguous with the mantle cavity of the squid. Surrounding the symbiotic bacteria and their supportive host cells are host tissues recruited into the light organ system, including a muscle-derived lens and thick reflector that appear to permit the squid to control the quality of bacterial light emission.

Introduction

Euprymna scolopes is a small (average adult mantle length, approximately 25 mm) benthic squid indigenous to Hawaii (Berry, 1912; Singley, 1983). As a nocturnal predator, it buries in the sand during the day and forages at night over shallow-water sand flats. *E. scolopes* shares with a number of other sepiolids the characteristic of culturing marine luminous bacteria in a symbiotic, light organ association (Kishitani, 1932; Boletzky, 1970; Herring *et al.*, 1981). The structural and functional relationships of the various tissues making up the complex light organ, as well as several behavioral observations (Moynihan, 1983; McFall-Ngai, pers. obs.), suggest that the luminescence is used by the squid in counterillumination (*i.e.*, production of ventrally directed luminescence to camouflage its silhouette against background light) and in startle displays (Herring *et al.*, 1981; Moynihan, 1983; McFall-Ngai, pers. obs.).

The present paper describes the anatomical and ultrastructural relationships of *E. scolopes* and its bacterial partner, *Vibrio fischeri*, in the adult light organ association. In providing a baseline understanding of the tissue relationships in the mature system, this report complements emerging studies on the initiation, development, and specificity of mutualistic associations between higher animals and bacteria (McFall-Ngai and Ruby, 1989; Ruby and McFall-Ngai, 1989).

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Materials and Methods

Specimens of *Euprymna scolopes* were collected with dipnets from shallow (<1 m) water in Kaneohe Bay on the island of Oahu, Hawaii, shortly after dusk during March 1988, 1989, and December 1989. The animals were transported to recirculating aquaria at the University of Hawaii, Manoa campus, or to running-seawater tables at the Hawaiian Institute of Marine Biology (University of Hawaii) on Coconut Island in Kaneohe Bay, where they were maintained until used in experiments.

Animals were fixed for light microscopy in 10% formaldehyde buffered with 0.5 *M* sodium phosphate, pH 7.4. They were then dehydrated through a graded ethanol series and infiltrated with propylene oxide before transfer to unaccelerated Spurr (Spurr, 1969). The samples remained in unaccelerated Spurr for one week at room temperature, followed by 24 h in accelerated Spurr at room temperature. Samples were then embedded in fresh accelerated Spurr in an oven at 67°C for 48 h.

The general construction of the light organ, and information from the literature on fixation procedures for transmission electron microscopy of similar tissues (Herring *et al.*, 1981), indicated that penetration of the light organ by fixatives might be a problem. Therefore, we conducted a series of experiments to determine the optimal fixation conditions. We fixed both whole and subdivided

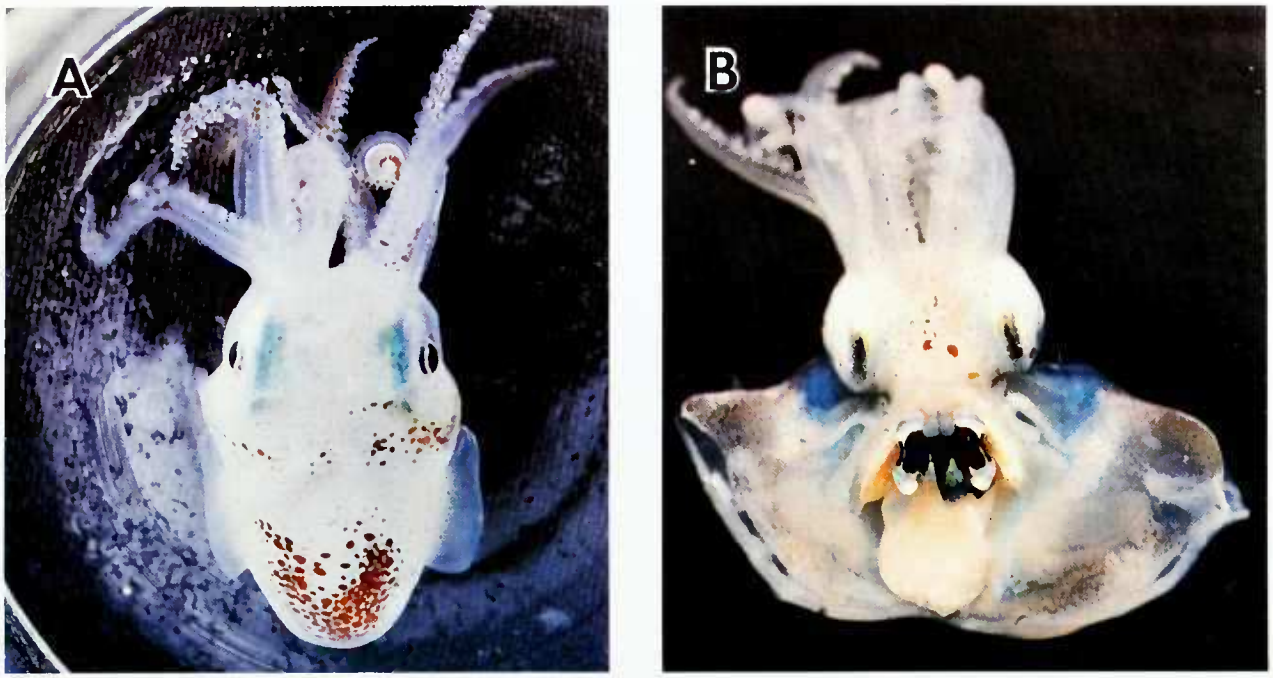


Figure 1. *Euprymna scolopes.* A. Dorsal view of adult specimen (mantle length approximately 20 mm). B. Ventral dissection of same specimen exposing the light organ. The light organ appears as the black and white kidney-shaped lobes overlaying the yellow digestive gland.

light organs from small and large specimens for either 60, 75, or 90 min, or for 6, 12, or 18 h. In addition, we tested the quality of ultrastructural detail obtained with the following fixatives: 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer with 0.40 *M*, 0.45 *M*, or 0.50 *M* NaCl, pH 7.4; and 2.5% glutaraldehyde in 0.2 *M* sodium phosphate buffer with 0.14 *M* NaCl, pH 7.4. All samples were postfixed in 1% osmium tetroxide in the fixative buffer and were processed similarly to those samples fixed for histology (see above).

Our experiments with tissue preparation for electron microscopy showed that the best preservation resulted from long fixation times (>6 h) in 0.1 *M* sodium cacodylate buffer with 0.45 or 0.50 *M* NaCl. Some components of the light organ showed enhanced fixation with 0.45 *M* NaCl while others showed similar improvements with 0.50 *M* NaCl (see figure legends).

Results

The fully developed light organ of *Euprymna scolopes* is a bilobed structure that occupies a significant portion of the mantle cavity (Fig. 1A, B). Ten specimens, ranging in size from 4.0 mm to 25 mm in dorsal mantle length (ML), were used for microscopy. The light organ ranged in anterior-posterior length from 1.5 mm in the smallest animal to 7.5 mm in the largest individual. Although ongoing studies in our laboratory have shown that the development of the *E. scolopes* light organ involves a set of complex stages, all components of the light organ are present and appear mature in juveniles as small as 4 mm ML.

Histological analysis of the light organ of this species (Fig. 2) revealed that the tissues and their anatomical relationships were similar to those described in the light organ system of another sepiolid, *Sepiola atlantica* (Her-

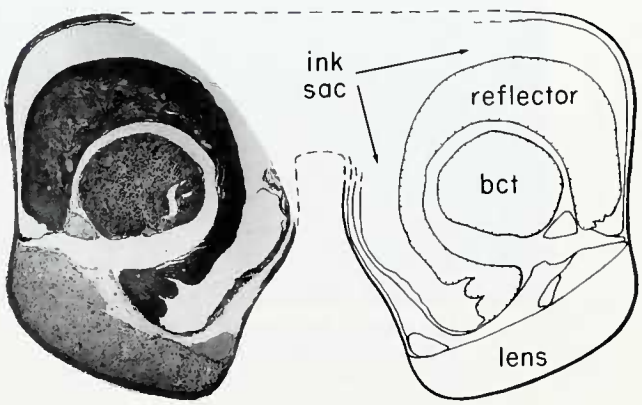


Figure 2. Light micrograph and opposing diagram of a typical 1-μm histological cross-section through the light organ of *Euprymna scolopes* showing the various associated tissues. The ink is lost from the ink sac during dissection, and shrinkage that occurs during fixation and embedding procedures causes the tissue containing the bacteria to pull away from the reflector. 20×. (bct, tissue containing bacteria).

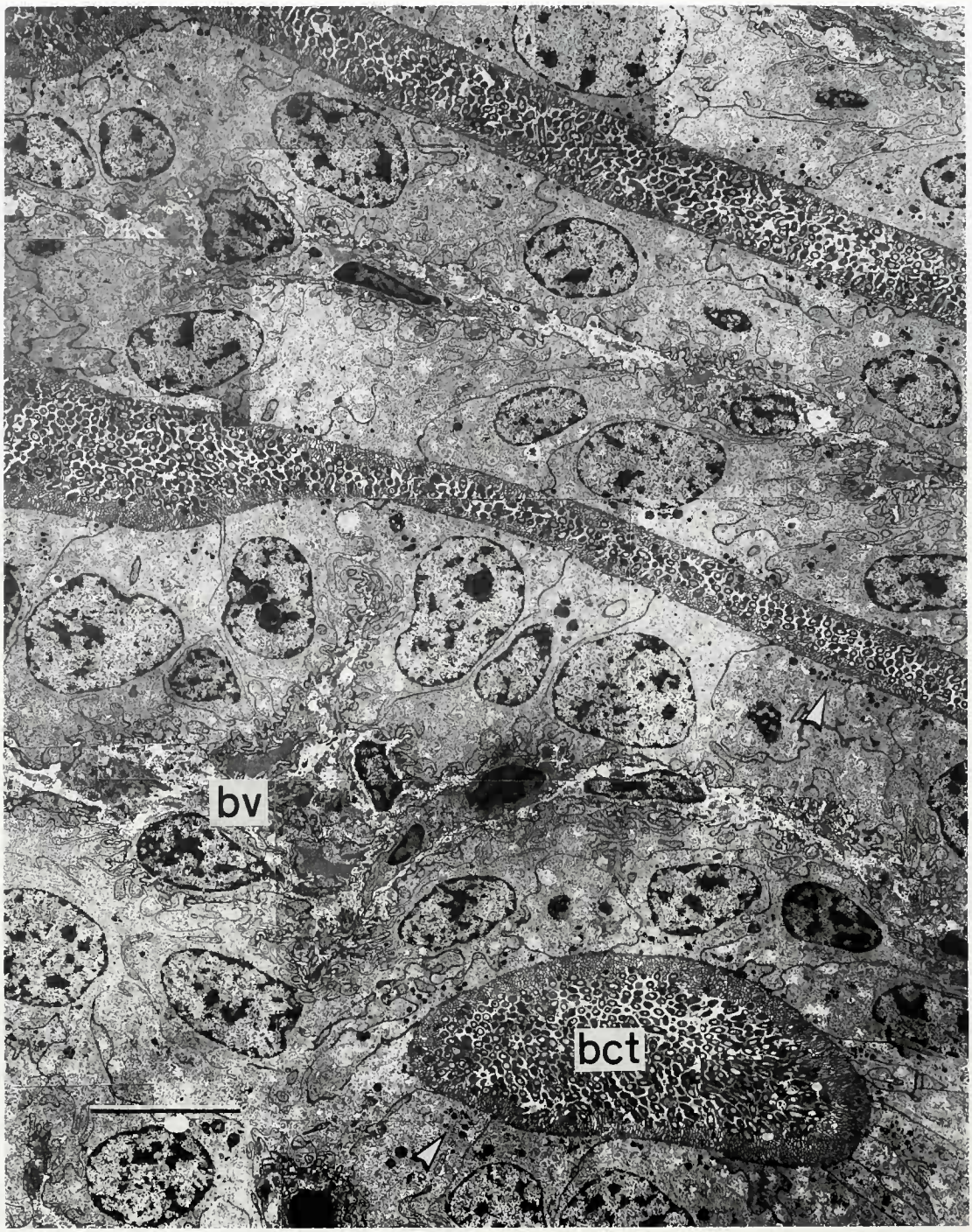


Figure 3. Composite transmission electron micrograph of the bacteria-containing central core of the light organ of *Euprymna scolopes*. Numerous electron-dense vesicles (arrows) are concentrated in portions of the host cell adjacent to the tubules that contain bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 10 μm. (bct, bacteria; bv, blood vessel)

ring *et al.*, 1981). The bacteria occur in animal tissue that is surrounded by a thick reflector, which is, in turn, surrounded by diverticula of the ink sac. In some preserved specimens, the medioventral portion of the reflector was pulled back, and ink in the ventral portions of the ink sac shunted medially (see Fig. 2). Histological analysis of a large number of specimens showed considerable variability in the positions of the reflector and ink sac in re-

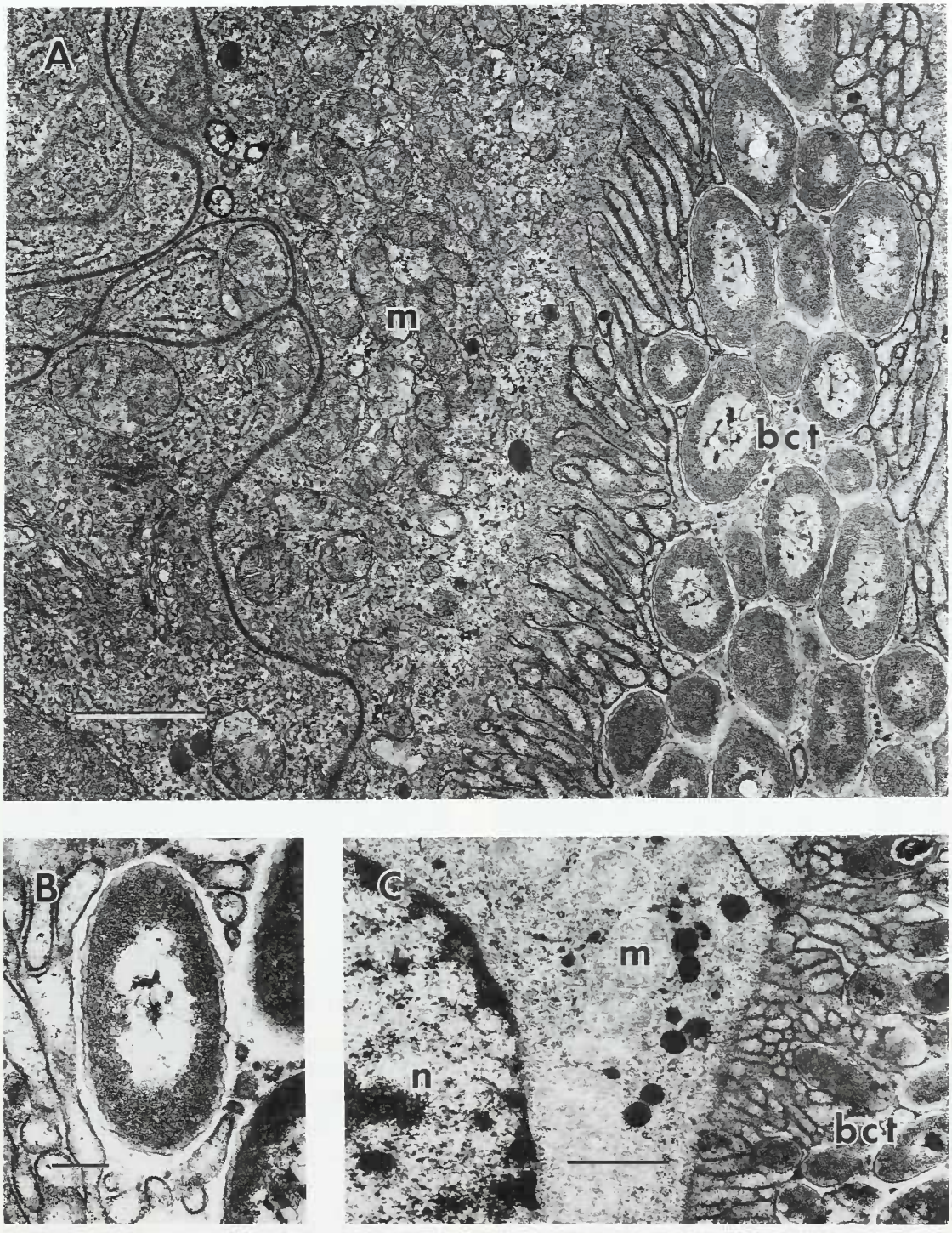


Figure 4. High magnification transmission electron micrographs of the light organ tissue that contains the bacteria. A. Abundant mitochondria occur in the host cell adjacent to the microvillous border lining the tubule that houses the bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.50 *M* NaCl. Bar scale = 1 μm. B. *Vibrio fischeri* cell as it appears in symbiosis with squid tissue. This single bacterium, although extracellular, appears almost completely surrounded by host cell membrane. Primary fixative is the same as above. Bar scale = 0.25 μm. C. The lower osmolarity of this buffer resulted in poor fixation of the mitochondria, but better fixation of the electron-dense vesicles, both of which appear in abundance in the portion of the animal cell adjacent to the microvillous border that lines the tubules containing bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 1 μm. (bct, bacteria; m, mitochondria; n, nucleus)

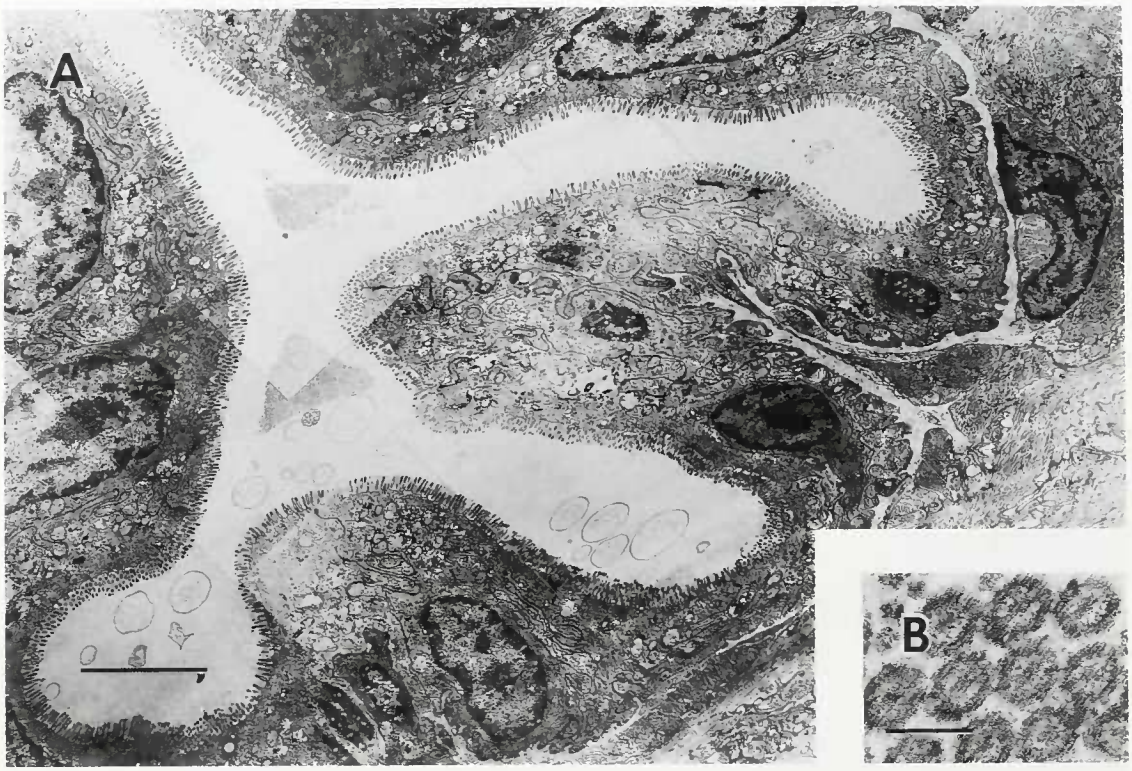
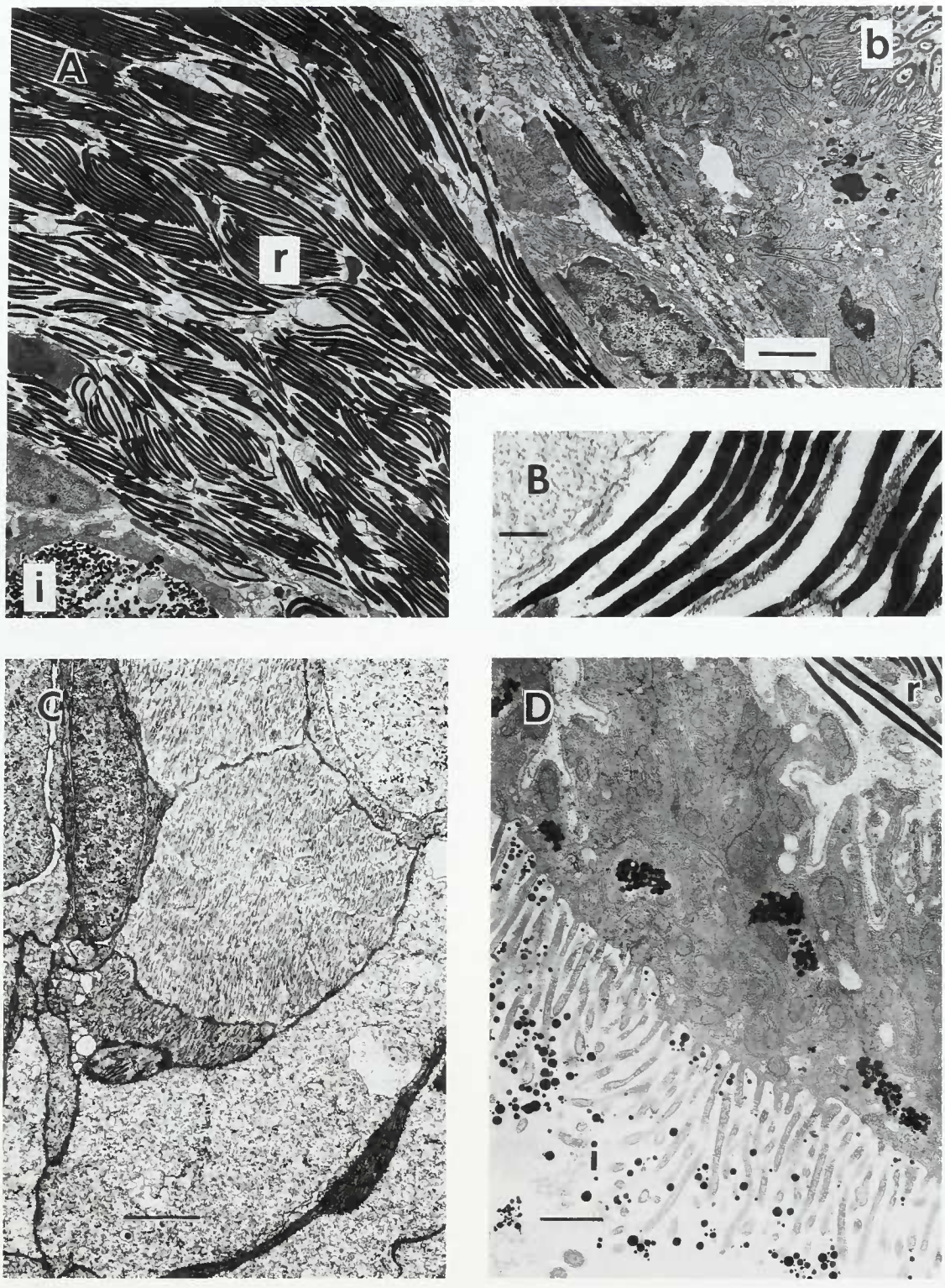


Figure 5. Composite electron micrograph of the ciliated duct of the light organ of *Euprymna scolopes*. A. The composite resulted from a transverse section of the light organ and shows the ciliated duct that leads to the lateral pore on each lobe. Branches of the duct shown in this micrograph are contiguous with the tubules that contain bacteria. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 5 μm. B. High magnification showing the cilia in cross-section. Primary fixative as above. Bar scale = 0.25 μm.

lation to the tissue that contains the bacterial symbionts. These data, coupled with observations of the behavior of light organ tissues of anesthetized, dissected animals, indicate that the expression of light is controlled by movements of both the reflector and ink sac. The entire ventral surface of the light organ is covered by a thick, transparent lens, the outer edge of which is continuous with the ink sac lining (Fig. 2). Microscopic examination of the intact light organ revealed a pore on the lateral face of each light organ lobe. Histological sections through this area revealed that the pore is continuous with the light organ tissue that contains bacteria (data not shown).

Low magnification transmission electron micrographs of light organ tissue that contains bacteria (Fig. 3) reveal that the bacteria occur in narrow channels, usually only a few bacteria in width, in most portions of the light organ. The narrow channels are surrounded by a single layer of animal cells, which are surrounded by a layer of blood vessels and other connective tissue elements. This pattern is repeated through the bacterial core tissue. Microvilli from the epithelial cells of the animal invest the bacterial culture. Electron-dense vesicles occur in the portions of the animal cells adjacent to the bacterial culture, suggesting the exchange of materials. Observations at higher magnifications of the animal cell/bacterium interface (Fig. 4) revealed that, in addition to electron-dense vesicles,

Figure 6. Accessory structures of the light organ of *Euprymna scolopes*. A. Transmission electron micrograph of the light organ with some of the structures associated with light modulation. This section is through a particularly narrow portion of the reflector (r) so that several layers of the system are viewed. The reflector is closely associated with the lining of the ink sac (see also 6D) and surrounds much of the central core tissue, which contains the bacterial symbionts. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.45 *M* NaCl. Bar scale = 2 μm. B. High magnification micrograph of the reflector showing the membrane bound platelets. Primary fixative same as above. Bar scale = 0.25 μm. C. Transmission electron micrograph of the lens exposing its lack of detailed cell structure but indicating its



development from muscle-derived tissue. Note the numerous, thin, aligned filaments filling most of the cells. Primary fixative used was 2.5% glutaraldehyde in 0.2 *M* sodium cacodylate buffer with 0.5 *M* NaCl. Bar scale = 2 μm. D. Transmission electron micrograph of the lining of the ink sac showing its ciliated ink-producing cells and its close association with the reflector. Primary fixative used was same as for 6A. Bar scale = 1 μm. (b, bacteria; i, ink sac; r, reflector)

high densities of mitochondria exist in portions of the animal cell adjacent to the bacteria. Tubules containing bacteria empty into a common, ciliated duct that is connected with the pore on the lateral face of each light organ lobe (Fig. 5). This duct provides a direct connection between the bacterial culture and the mantle cavity of the animal.

The tissue containing bacteria is surrounded by reflective tissue, which is itself enclosed by the ink sac (Fig. 6A). The reflector is made up of cells containing platelets oriented perpendicularly to the bacterial culture, and has a similar structure to that reported for the reflective tissues of other cephalopods (Arnold *et al.*, 1974; Brocco and Cloney, 1980; Cloney and Brocco, 1983). The width of the reflector is several dozen to hundreds of platelets thick, depending on the location within the light organ. These electron-dense reflector platelets, which appear to be membrane bound (Fig. 6B), averaged 100 nm wide with cytoplasmic spacing that varies from 50 nm to 200 nm. Because of shrinkage during preparation, however, this may not represent the actual spacing. The ventral portion of the reflector abuts the lens of the light organ (see Fig. 2), the ultrastructural characteristics of which suggested that it was derived from muscle tissue (Fig. 6C). The cells of the lens had little structural detail except for the presence of numerous, thin, aligned elements. The ink sac lining appeared as a mitochondrion-rich tissue with electron-dense vesicles, presumably packaged ink (Fig. 6D).

Discussion

Progress toward an understanding of developmental processes in higher animal/bacterial mutualisms has been slow because of the lack of tractable experimental systems. Unlike plant mutalisms, such as the leguminous plant/*Rhizobium* symbiosis (Long, 1989), animal mutualisms usually involve a variety of different species of microorganisms in a single host, or are characterized by a host that cannot live axenically or by symbionts that cannot be cultured. One of the few higher animal/bacterial mutualisms the study of which does not suffer from these drawbacks, is the symbiotic relationship between the sepiolid squid, *Euprymna scolopes*, and its luminous bacterial symbiont, *Vibrio fischeri*. The squid host, which can be raised in the laboratory (Arnold *et al.*, 1972; pers. obs.), hatches without its luminous symbiont (Wei and Young, 1989). *V. fischeri*, which occurs freeliving in the water and is readily culturable (Ruby and McFall-Ngai, 1989; Boettcher and Ruby, 1990), is picked up by the newly hatched squid within hours after hatching (Wei and Young, 1989).

A prerequisite to the studies of the development of the light organ is a description of the morphology and anatomy of the adult association. Although the adult light organ of *E. scolopes*, described here, is similar to that of other sepiolids (Kishitani, 1932; Herring *et al.*, 1981), some ultrastructural differences have emerged. Herring *et al.* (1981) reported that the bacteria of another sepiolid squid, *Sepiola atlantica*, are loosely associated with the animal cells, which lack microvilli. In contrast, the bacterial culture in the light organ of *E. scolopes* is in intimate contact with the microvillous border of the host cells. Caution must be exercised in interpreting these differences, which could be due to differences in the quality of fixation.

The high concentrations of mitochondria in the portions of the animal cells adjacent to the bacteria may be of particular significance in the physiology and metabolic dynamics of the light organ association. Monocentrid fishes also have light organs containing an abundance of mitochondria in the cells next to the bacterial symbionts (Tebo *et al.*, 1979). Under laboratory culture conditions of low oxygen, *Vibrio fischeri* grows poorly, but luminesces brightly and excretes pyruvate (Ruby and Nealson, 1976; Nealson and Hastings, 1977). The ultrastructure of the monocentrid light organ and the physiology of the bacteria in culture has led to a model for this symbiosis (Nealson, 1979). The model holds that pyruvate excreted by the bacteria fuels the mitochondria, the respiratory activity of which keeps the oxygen tension low around the bacterial culture, thus promoting the slow growth but high luminescence of the bacteria in the association. However, while the light organ tubule cells of *E. scolopes* also have high densities of mitochondria, the symbiotic strain of *V. fischeri* they surround does not show enhanced luminescence under low oxygen tensions (Boettcher and Ruby, 1990). Thus, physiological behavior of the *E. scolopes* bacteria in culture and the ultrastructural characteristics of the light organ are inconsistent with the model developed for the monocentrid fish symbiotic association.

During the development and ontogeny of a complex light organ, such as that of *Euprymna scolopes*, tissues must be recruited and modified to form the various components of the organ. Not only must tissue be adapted so that the squid can efficiently use the light produced by the bacteria, but the light organ and the bacterial culture must be supported by recruited vascular and nervous tissue. Further, the squid host must produce a site that promotes the growth and luminescence of the native symbiotic bacterium, while excluding other bacterial species. How these processes are orchestrated to create the complex adult structure, and the part played by the bacteria in the morphogenesis of the light organ, should be revealed through experimental manipulations of the developing system.

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